Vol. 79, No. 12

T Cells with a CD4+CD25+ Regulatory Phenotype Suppress In Vitro Proliferation of Virus-Specific CD8+ T Cells during Chronic Hepatitis C Virus Infection

Tobias Boettler,[1]† Hans Christian Spangenberg,[1]† Christoph Neumann-Haefelin,[1]
Elisabeth Panther,[1] Simonetta Urbani,[2] Carlo Ferrari,[2] Hubert E. Blum,[1]
Fritz von Weizsäcker,[1] and Robert Thimme[1]*

*Department of Medicine II, University Hospital Freiburg, Freiburg, Germany,[1] and Division of
Infectious Diseases and Hepatology, University of Parma, Parma, Italy[2]*

Chronic hepatitis C virus (HCV) infection is associated with impaired proliferative, cytokine, and cytotoxic effector functions of HCV-specific CD8+ T cells that probably contribute significantly to viral persistence. Here, we investigated the potential role of T cells with a CD4+CD25+ regulatory phenotype in suppressing virus-specific CD8+ T-cell proliferation during chronic HCV infection. In vitro depletion studies and coculture experiments revealed that peptide specific proliferation as well as gamma interferon production of HCV-specific CD8+ T cells were inhibited by CD4+CD25+ T cells. This inhibition was dose dependent, required direct cell-cell contact, and was independent of interleukin-10 and transforming growth factor beta. Interestingly, the T-cell-mediated suppression in chronically HCV-infected patients was not restricted to HCV-specific CD8+ T cells but also to influenza virus-specific CD8+ T cells. Importantly, CD4+CD25+ T cells from persons recovered from HCV infection and from healthy blood donors exhibited significantly less suppressor activity. Thus, the inhibition of virus-specific CD8+ T-cell proliferation was enhanced in chronically HCV-infected patients. This was associated with a higher frequency of circulating CD4+CD25+ cells observed in this patient group. Taken together, our results suggest that chronic HCV infection leads to the expansion of CD4+CD25+ T cells that are able to suppress CD8+ T-cell responses to different viral antigens. Our results further suggest that CD4+CD25+ T cells may contribute to viral persistence in chronically HCV-infected patients and may be a target for immunotherapy of chronic hepatitis C.

Hepatitis C virus (HCV) is a parenterally transmitted hepatotropic RNA virus that causes acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Infection with HCV affects an estimated 200 million people worldwide (21). It is widely accepted that cellular immune responses play an important role in the immunopathogenesis of HCV infection since viral clearance is associated with vigorous and multispecific HCV-specific T-cell responses during acute infection (8, 11, 14, 23, 26, 41). In contrast, in chronically infected patients, HCV-specific T-cell responses are generally weak, narrowly focused, and often dysfunctional (7, 20, 32, 43, 47). Indeed, several investigators have clearly shown that HCV-specific CD8+ T cells have functional defects during chronic infection, as indicated by impaired gamma interferon (IFN-γ) production and in vitro proliferation (14, 47). While the mechanisms responsible for the dysfunctions of HCV-specific T cells in chronically infected patients remain unclear, recent studies suggest a major contribution of regulatory CD4+ and CD8+ T cells (1, 2, 9, 36).

Currently, the best-characterized regulatory T cells are CD4+CD25+ T cells (30). These cells recognize peptides presented by major histocompatibility complex class II molecules and can suppress host immune responses and modulate immune responses in the setting of autoimmune diseases, allergy, transplantation, infectious diseases, and antitumor immunity (33, 34). Two major populations of CD4+CD25+ cells can be distinguished: naturally occurring regulatory T cells (Tregs) and induced Treg cells. Naturally occurring Treg cells consist of CD4+ cells that mature in the thymus to regulatory T cells. They represent 5 to 10% of peripheral CD4+ T cells and constitutively express CD25. Induced Treg cells have acquired their suppressive activity during activation in vitro or in vivo and are derived from CD4+CD25− T cells (4, 45). The mechanisms underlying the suppressive functions of Treg cells are not completely understood. Some studies have indicated that direct cell-cell contact is required whereas others have suggested that cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGF-β) may play an important role in the suppressive function of Treg cells (17, 29, 30, 34).

An increasing number of studies indicates a potential role of Treg cells in the control of virus-specific T-cell responses during acute and chronic viral infections (27). For example, the impairment of IFN-γ secretion of CD8+ T cells by Treg cells has been described during persistent retroviral infection in mice, whereas proliferative capacity of virus-specific CD8+ T cells was unaffected (9). Recent studies have also suggested that Treg cells suppress virus-specific CD8+ T-cell effector functions in chronic human viral infections such as HCV, human immunodeficiency virus, and cytomegalovirus (1, 5, 19, 36).

---

* Corresponding author. Mailing address: Department of Medicine II, University Hospital Freiburg, Hugstetter Strasse 55, D-79106 Freiburg, Germany. Phone: 49-761-270 3280. Fax: 49-761-270 3372. E-mail: thimme@med1.ukl.uni-freiburg.de.
† Equal contribution.

TABLE 1. Patient data

Patient group	Patient no.	Age (yr)	Gender	ALT^a (U/liter)	Anti-HCV antibody	HCV PCR	Genotype	Viral load (genome equivalents/ml)	Recovery
Chronic HCV									
	C1	38	F	59	+	+	1	500,000	
	C2	41	M	109	+	+	1	29,000,000	
	C3	25	M	62	+	+	1	150,000	
	C4	47	F	83	+	+	1	1,700,000	
	C5	49	F	14	+	+	1	2,000,000	
	C6	30	M	41	+	+	1	Not done	
	C7	39	M	100	+	+	1	900,000	
	C8	46	M	150	+	+	1	5,000	
Resolved HCV									
	R1	44	F		+	−			Therapy
	R2	31	M		+	−			Spontaneous
	R3	43	M		+	−			Therapy
	R4	49	M		+	−			Spontaneous
	R5	53	M		+	−			Spontaneous
	R6	36	M		+	−			Therapy
	R7	53	F		+	−			Therapy
	R8	27	F		+	−			Spontaneous

^{a} ALT, alanine aminotransferase level.

In this study, we show on a single epitope level using well-described HLA-A2 epitopes and tetramer technology that proliferation of virus-specific CD8$^+$ T cells is inhibited by Treg cells in a dose-dependent manner, that this suppression requires direct cell-cell contact, that this inhibition is independent of interleukin (IL)-10 and transforming growth factor beta (TGF-β), that it is not restricted to virus-specific CD8$^+$ T cells targeting the persisting virus, and that the suppression of virus-specific CD8$^+$ T cells seems to be specific for chronic infection since the degree of suppression varies significantly between patients with chronic HCV infection versus persons with resolved HCV infection and healthy blood donors.

MATERIALS AND METHODS

Subjects. Blood samples from HLA-A2-positive healthy blood donors, chronically HCV-infected patients, and patients with resolved HCV infection were obtained after informed consent. Peripheral blood mononuclear cells (PBMC) were isolated from EDTA anticoagulated blood samples through Ficoll-Paque PLUS density gradient (Amersham, Uppsala, Sweden) or lymphocyte separation medium (PAA, Vienna, Austria). After isolation, cells were washed two times in phosphate-buffered saline (PBS) (Gibco, Auckland, N.Z.). The clinical and virological data of the patients studied are summarized in Table 1. All experiments described below were performed in duplicates.

CD4 depletion. Selection of non-CD4$^+$ cells was performed either by using CD4 Dynabeads (Dynal Biotech, Oslo, Norway) or by indirect magnetic labeling of non-CD4$^+$ T cells using MicroBeads (CD4$^+$CD25$^+$ regulatory T-cell isolation kit, Miltenyi Biotec, Auburn, CA).

Dynabeads. Depletion of CD4$^+$ cells with Dynabeads was performed according to the manufacturer's instructions. Briefly, up to 10^7 cells were resuspended in 1 ml PBS containing 1% fetal bovine serum (FCS); 144 μl Dynabeads per 10^7 cells were washed two times with 1 ml PBS containing 1% FCS and transferred to the resuspended cells. Cells were incubated for 30 min at 4°C under slow rotation, followed by separation through a Dynal MPC magnet.

MicroBeads. PBMC were incubated for 10 min with 90 μl of MACS buffer (PBS, pH 7.2, containing 0.5% bovine serum albumin [BSA] and 2 mM EDTA) and 10 μl CD4$^+$ T-cell biotin-antibody cocktail (antibody against CD8, CD14, CD16, CD19, CD36, CD56, CD123, T-cell receptor γ/δ, and glycophorin A) per 10^7 cells. Cells were washed and incubated with 20 μl antibiotin MicroBeads per 10^7 cells. After 15 min of incubation cells were washed, resuspended in MACS buffer and applied to a magnetic column (LD) on a MidiMACS separation unit. Unlabeled CD4$^+$ cells as well as labeled CD4-depleted PBMC were collected. Cell populations were analyzed by fluorescence-activated cell sorter (FACS) analysis and were 90 to 99% pure (data not shown).

CD25 selection. The CD4$^+$ cell population was separated into CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ (Treg) cells with anti-CD25 MicroBeads. Cells were incubated for 15 min in 90 μl MACS buffer and 10 μl anti-CD25 MicroBeads per 10^7 cells. After washing, cells were resuspended in MACS buffer and applied to an MS column on a MiniMACS separation unit. Both resulting populations were collected. The purity of the CD4$^+$CD25$^+$ T cells ranged from 82 to 97% (data not shown).

Synthetic peptides. HCV-derived peptides previously shown to be HLA-A2-restricted HCV epitopes were synthesized with a free N and C terminus (Biosynthan, Berlin, Germany). The amino acid sequences of the HLA-A2-restricted HCV and influenza virus epitopes are shown in Table 2.

Antigen-specific cell proliferation. We resuspended 2 × 10^6 PBMC or CD4-depleted PBMC in 1 ml RPMI (Gibco, Auckland, N.Z.) containing 10% FCS, 1% streptomycin/penicillin, and 1.5% HEPES buffer (1 M), stimulated with 10 μg/ml of synthetic HCV or influenza virus peptide and 0.5 μg/ml anti-human CD28 (BD PharMingen, San Jose, CA) and cultured on a 48-well plate in the absence or presence of CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ T cells as indicated. In some experiments 20 U/ml and 200 U/ml human recombinant IL-2 (Hoffmann La Roche, Inc., Basel, Switzerland) was added on day 0 or day 3 to the culture. In addition, in selected experiments CD4$^+$CD25$^+$ T cells were irradiated (30 Gy) prior to adding to the culture. After incubation for 7 days, cells were stained for antigen-specific responses. FACS analysis was performed on a BD FACSCalibur flow cytometer using Cell Quest software (BD Biosciences).

Tetramer staining. We blocked 0.2 to 0.3 × 10^6 cells per well with immunoglobulin G1 pure (BD PharMingen, San Jose, CA) for 20 min and stained with anti-CD8-phycoerythrin (CD8 PE, Miltenyi Biotec, Auburn, CA or CD8 Cy7-PE, BD PharMingen, San Jose, CA) antibody for 20 min on a 96-well plate at 4°C. Cells were washed three times with PBS containing 1% FCS and incubated with allophycocyanin (APC)-conjugated HLA-A2 tetramers (National Institutes of Health tetramer facility, Atlanta, MD, for HCV, and ProImmune, Oxford, United Kingdom, for influenza virus) corresponding to the HCV and influenza virus peptides as shown in Table 2. Incubation was performed at 37°C and 5% CO$_2$ for 20 min. Cells were washed three times and fixed in 100 μl CellFIX (BD PharMingen, San Jose, CA) per well.

TABLE 2. HLA-A2-restricted epitopes

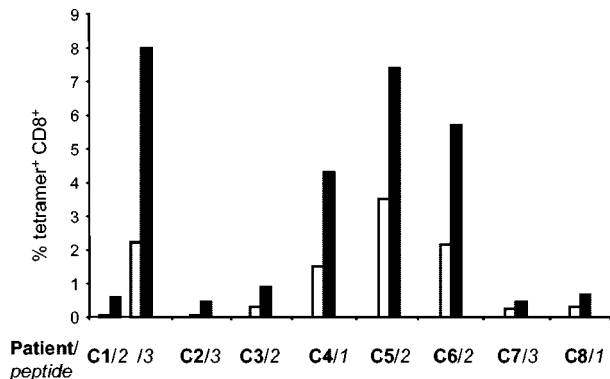| Virus	Peptide	Amino acid sequence	Protein and positions
HCV	1	CINGVCWTV	NS3 1073
	2	KLVALGINAV	NS3 1406
	3	ALYDVVTKL	NS5 2594
Influenza virus	4	GILGFVFTL	Matrix 58

FIG. 1. Depletion of CD4$^+$ cells enhances HCV-specific CD8$^+$ T-cell responses. Tetramer staining of a T-cell line after 7 days in culture. PBMC (white bars) and PBMC depleted of CD4$^+$ cells (black bars) were stimulated with HCV peptides and cultured for 7 days. In all cases, in vitro stimulated T-cell lines showed an enhanced expansion after depletion of CD4$^+$ cells.

Intracellular IFN-γ staining. Procedures were performed essentially as described previously (7). Briefly, cells (0.2×10^6 per well, 96-well plate) were stimulated with peptides (10 μl/ml) in the presence of 50 U/ml human recombinant IL-2 (Hoffmann La Roche, Inc., Basel, Switzerland) and 1 μl/ml brefeldin A (BD PharMingen, San Jose, CA). After 5 h incubation (37°C, 5% CO$_2$), cells from each well were blocked and stained with antibodies against CD8 (Miltenyi Biotec, Auburn, CA). Prior to staining with intracellular antibodies against IFN-γ (BD PharMingen, San Jose, CA), cells were fixed and permeabilized by adding Cytofix/Cytoperm (BD PharMingen, San Jose, CA). Cells were washed three times and fixed in 100 μl CellFIX (BD PharMingen, San Jose, CA) per well.

CFSE. We incubated 10^7 cells (CD4-depleted PBMC) with 5 μmol/liter CFSE (5- and 6-carboxyfluorescein diacetate succinimidyl ester, Molecular Probes, Eugene, OR) for 10 min at 37°C (24). After incubation, cells were washed three times with complete medium and cultured in the presence of CD4$^+$CD25$^+$ cells as indicated. Stimulation was performed as described above. Cells were stained with CD8 Cy7-PE and APC-conjugated HLA-A2 tetramers or CD4-APC antibody.

Transwell assay and cytokine neutralizing assays. CD4-depleted PBMC were cultured with or without CD4$^+$CD25$^+$ cells together in one well or separated through a microporous membrane with 0.4 μm pore size (Transwell, Corning Inc., Acton, MA). In the transwell assay, the CD4-depleted PBMC were put into the outer compartment of a 24-well plate, the Treg cells into the inner compartment. For neutralizing assays, CD4-depleted PBMC were cultured with CD4$^+$CD25$^+$ cells in the presence of 0.5 μg/ml mouse anti-human IL-10 (R&D Systems, Minneapolis, MN), 10 μg/ml of chicken anti-human TGF-β (R&D systems), or isotype controls. Culture, stimulation and staining procedures were performed as described above.

Antibodies. CD8-PE, CD4-fluorescein isothiocyanate (FITC), CD4-APC, CD25-PE (all Miltenyi Biotec, Auburn, CA) antibodies as well as CD8-Cy7-PE, isotype FITC, PE, APC, and Cy7-PE (BD PharMingen, San Jose, CA) were used according to the manufacturers' instructions.

Statistical analysis and tetramer calculation. Statistical analyses were performed using the Mann-Whitney test. All tetramer data are shown as the percentage of tetramer-positive CD8$^+$ T cells in relation to all CD8$^+$ T cells. To express the percentage of CD4$^+$CD25$^+$ cell-mediated inhibition after 7 days in culture (as shown in Fig. 5) the following calculation was performed: $100 -$ (percentage of tetramer-positive CD8$^+$ T cells in the presence of CD4$^+$CD25$^+$ cells divided by the percentage of tetramer-positive CD8$^+$ T cells in the presence of CD4$^+$CD25$^-$ cells) $\times 100$.

RESULTS

Depletion of CD4$^+$ cells augments in vitro expansion of virus-specific CD8$^+$ T-cell responses during chronic HCV infection. CD4$^+$ helper T cells are required for the priming and expansion of CD8$^+$ T cells (18). By contrast, 5 to 10% of CD4$^+$ cells, CD4$^+$ Treg cells, can profoundly suppress host immune responses. To determine which function of CD4$^+$ cells, help

versus suppression, dominates during chronic HCV infection, we depleted CD4$^+$ cells from PBMC obtained from chronically HCV-infected HLA-A2-positive patients before the cells were stimulated with HLA-A2-derived HCV peptides. Importantly, as shown in Fig. 1, the depletion of CD4$^+$ cells prior to stimulation with HCV peptides markedly enhanced the HCV-specific CD8$^+$ T-cell response after 7 days in vitro peptide stimulation. In one patient, this effect was even observed against two different HCV peptides (patient 1, Fig. 1). These results show that CD4$^+$ cells are not required for the expansion of virus-specific CD8$^+$ T cells in vitro, but suggest that CD4$^+$ cells suppress the expansion of HCV-specific CD8$^+$ T cells during chronic infection.

Suppression of CD8$^+$ T-cell expansion by CD4$^+$CD25$^+$ Treg cells. Next we analyzed whether the CD4$^+$ cell-mediated HCV-specific CD8$^+$ T-cell suppression may be mediated by CD4$^+$CD25$^+$ Treg cells since this population has previously been shown to inhibit the induction of effector CD8$^+$ T-cell functions (6, 31). CD4$^+$CD25$^+$ Treg cells as well as CD4$^+$CD25$^-$ cells were purified from the PBMC of the chronically HCV-infected patients. The representative results of three patients in Fig. 2A and B show that addition of autologous CD4$^+$CD25$^+$
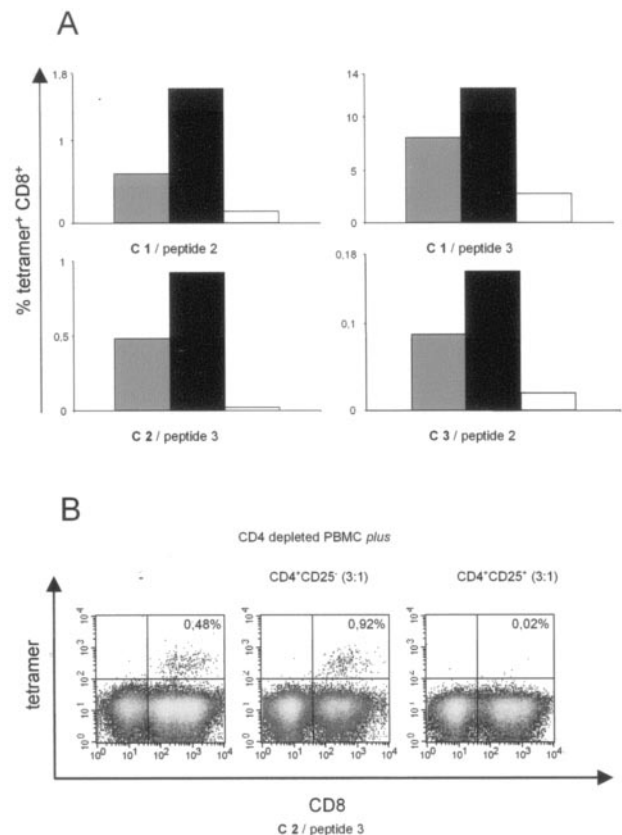

FIG. 2. CD4$^+$CD25$^+$ regulatory T cells suppress HCV-specific CD8$^+$ T-cell response. (A) PBMC depleted of CD4$^+$ cells were cultured alone (grey bars), in the presence of CD4$^+$CD25$^-$ (black bars) or CD4$^+$CD25$^+$ (white bars) for 7 days. Cells were stained with HLA-A2 tetramers. (B) PBMC depleted of CD4$^+$ cells were cultured alone, in the presence of CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ for 7 days. Representative density plots of cells stained with tetramer and anti-CD8 are shown.
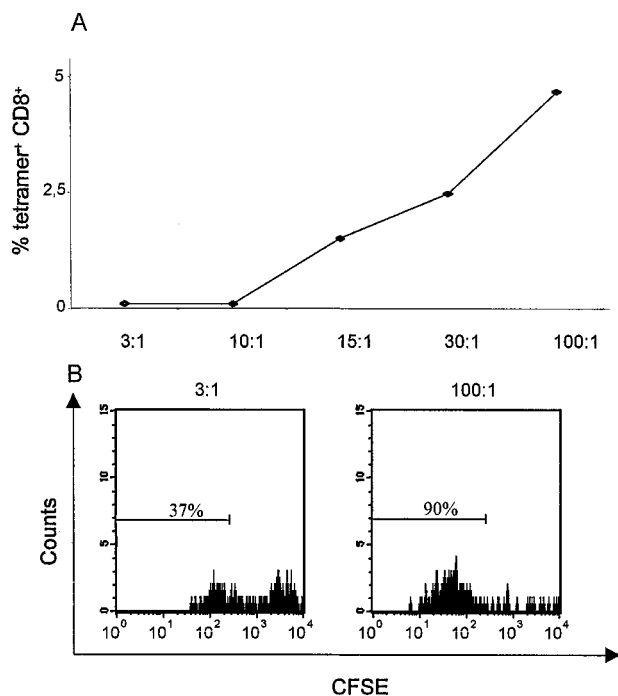
A

B

effect of Treg cells was clearly dose dependent, since decreasing numbers of CD4$^+$CD25$^+$ Treg cells were associated with less suppressive activity. CFSE labeling revealed that the suppression was largely due to inhibition of proliferation because significantly less cells became CFSE low in the presence of a high number of Treg cells (ratio, 3:1) versus a low number (ratio, 100:1) (Fig. 3B).

Suppressive activity of CD4$^+$ Treg cells is cell-cell contact dependent but independent of IL-10 and TGF-β. Depending on the model studied, T-cell suppression mediated by Treg cells has been reported to be cell-cell contact dependent, IL-10 dependent, and/or TGF-β dependent (30). To determine whether Treg cell-mediated suppression of HCV-specific CD8$^+$ T-cell proliferation occurred via cell-cell contact or cytokines, we performed transwell experiments and cocultured CD4-depleted PBMC with Treg cells in the presence of antibodies directed against IL-10 and TGF-β. As shown in Fig. 4A, Treg cells, when cultured in the inner well containing only medium, did not inhibit the proliferative activity of CD8$^+$ T cells cultured in the outer well containing the same medium. By contrast, the coculture of Treg cells and CD4-depleted PBMC in the outer

FIG. 3. Treg cells inhibit proliferation of HCV specific tetramer-positive CD8$^+$ T cells. (A) CD4-depleted PBMC were stimulated with HCV peptide 3 prior to culture with decreasing numbers of CD4$^+$CD25$^+$ regulatory T cells (CD4-depleted PBMC: Treg cells). After 7 days in culture, cells were tested for virus-specific responses by tetramer staining. (B) CD4-depleted PBMC were labeled with CFSE- and stimulated with HCV peptide 3 prior to culture with high (ratio 3:1) or low (ratio 100:1) numbers of CD4$^+$CD25$^+$ regulatory T cells. Representative histograms are shown. The cells are gated on CD8$^+$ HCV tetramer positive cells.

Treg cells to CD4-depleted PBMC markedly impaired the expansion of HCV-specific CD8$^+$ T cells after 7 days in vitro peptide specific stimulation. By contrast, we observed an enhanced virus-specific CD8$^+$ T-cell expansion in the presence of CD4$^+$CD25$^-$ T cells (Fig. 2A and B), indicating the presence of helper functions (e.g., due to cytokine secretion or antigen presentation) within this cell population. It is important to note, however, that the number of cells recovered after 1 week of coculture with CD4$^+$CD25$^+$ Treg cells or CD4$^+$CD25$^-$ was very similar. Intracellular IFN-γ staining was performed to test the effect of both CD4$^+$ populations on the function of CD8$^+$ cells. Treg cells also suppressed peptide-specific IFN-γ production of the expanded cells, whereas phorbol myristate acetate-stimulated IFN-γ production was largely unaffected (data not shown). Taken together, our CD4 depletion (Fig. 1) and coculture experiments (Fig. 2) suggest that the CD8$^+$ T-cell-mediated immune response to HCV is significantly limited by the action of Treg cells.

Treg cells inhibit proliferation of HCV-specific CD8$^+$ T cells in a dose-dependent manner. To further investigate the suppressive effects of Treg cells on the proliferative capacity of HCV-specific CD8$^+$ T cells at the single-cell level, we stained CD4-depleted PBMC with the fluorescent dye CFSE and determined the CFSE content of tetramer-positive cells after 7 days in culture in the presence of decreasing numbers of CD4$^+$CD25$^+$ Treg cells. As shown in Fig. 3A, the suppressive
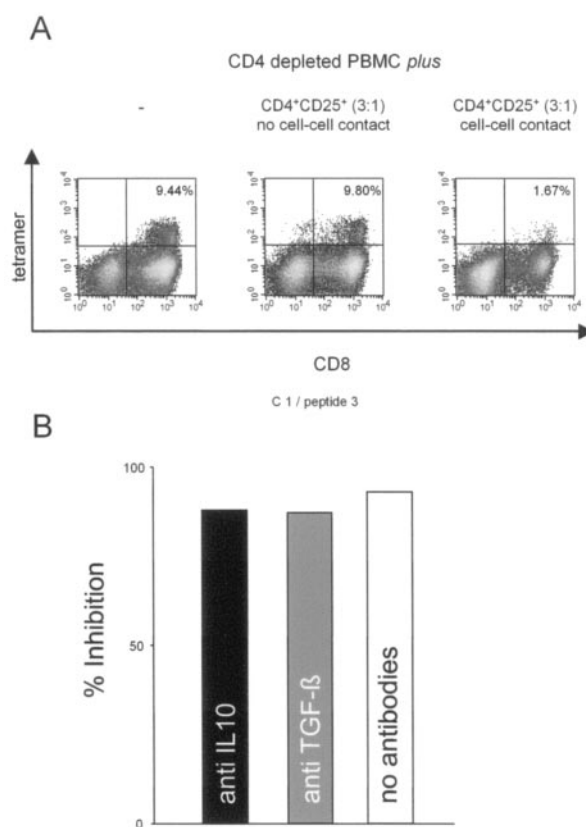
A

B

FIG. 4. Treg cell-mediated suppression is cell-cell contact dependent but independent of IL-10 and TGF-β. (A) PBMC were depleted of CD4$^+$ cells and cultured alone (left), in the presence of CD4$^+$CD25$^+$ regulatory T cells but separated through a microporous membrane (no cell-cell contact) (middle), and unseparated together in one well (cell-cell contact) (right). (B) Addition of neutralizing antibodies against IL-10 and TGF-β had no effect on the suppressive capacity of CD4$^+$CD25$^+$ regulatory T cells. Data are the means of duplicate wells.
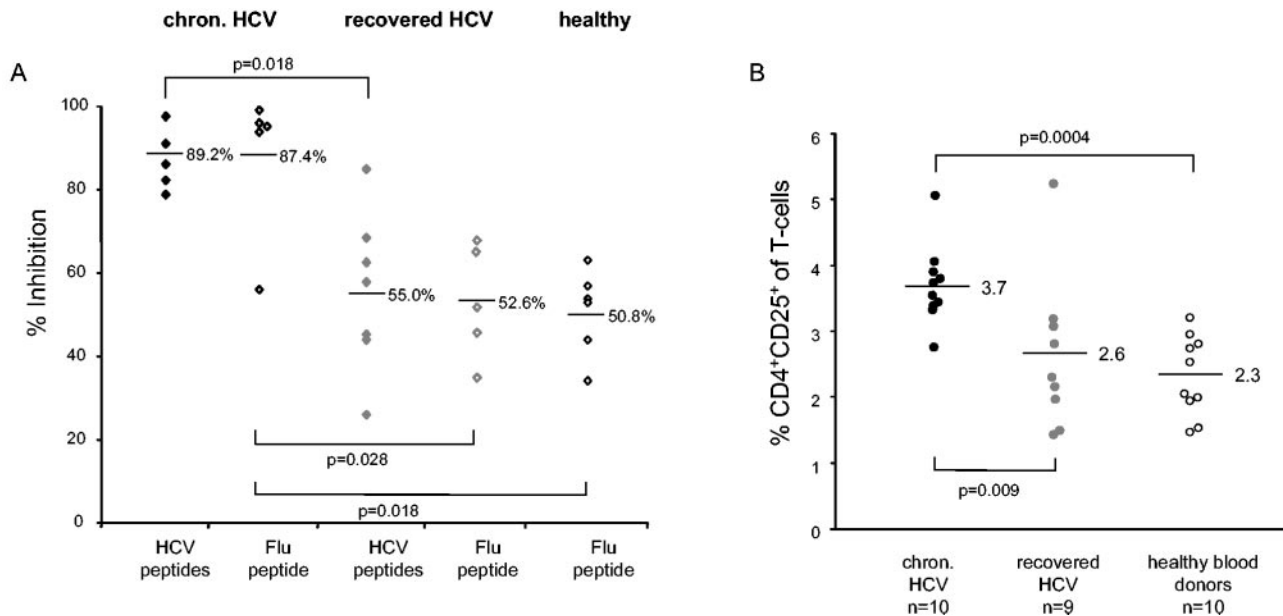
FIG. 5. Inhibition of HCV and influenza virus-specific CD8$^+$ T-cell proliferation by Treg cells in chronically HCV-infected patients and healthy blood donors. (A) T-cell lines from HCV-infected patients were tested for Treg-mediated suppression (ratio 3:1) of HCV-specific (patients C1 [two epitopes], C2, C3, and C4) and influenza virus-specific (patients C1, C2, C3, C4, and C5) CD8$^+$ T-cell proliferation after 7 days of antigen-specific stimulation by tetramer staining. To calculate the percentage of CD4$^+$CD25$^+$ cell-mediated inhibition after 7 days in culture the following formula was used: $100 -$ (percentage of tetramer-positive CD8$^+$ T cells in the presence of CD4$^+$CD25$^+$ cells divided by the percentage of tetramer-positive CD8$^+$ T cells in the presence of CD4$^+$CD25$^-$ cells) $\times$ 100. Patients with resolved HCV infection and healthy blood donors were tested in the same way for Treg-mediated suppression of HCV-specific (patients R1, R2 [two epitopes], R3, R7, and R8 [two epitopes]) and influenza virus-specific (patients R2, R3, R4, R5, and R6 and healthy blood donors) CD8$^+$ T-cell proliferation. Importantly, a significant difference was observed between the inhibition of HCV-specific and influenza virus-specific CD8$^+$ T cells in chronically HCV-infected patients compared to patients with resolved HCV infection and healthy blood donors (only influenza virus). (B) Frequency of CD4$^+$CD25$^+$ T cells in the peripheral blood of chronically HCV-infected patients, patients with resolved HCV infection, and healthy blood donors.

well led to a strong suppression of HCV-specific CD8$^+$ T-cell expansion after 7 days peptide-specific stimulation (Fig. 4A).

Noteworthy, proliferation of Treg cells is not required since the same suppression of virus-specific CD8$^+$ T cells was observed when Tregs were irradiated prior to adding them to the culture (data not shown). Moreover, coculture of CD4-depleted PBMC with Treg cells in the presence of antibodies directed against IL-10 or TGF-β had no effect on the suppressive activity of regulatory T cells (Fig. 4B). These results show that cell-cell contact is required for the suppressive activity of Treg cells and that it is independent of cytokines such as TGF-β and IL-10. Finally, the addition of exogenous IL-2 to the culture did not revert the inhibitory effect of Treg cells (data not shown).

Enhanced suppression of virus-specific CD8$^+$ T-cell proliferation in chronically HCV-infected patients compared to patients with resolved HCV infection or healthy blood donors. To determine the specificity of the Treg-mediated suppression of HCV-specific CD8$^+$ T cells in chronically HCV-infected patients, we compared the suppressive effects of Treg cells on the proliferative capacity of HCV-specific CD8$^+$ T cells versus influenza virus-specific CD8$^+$ T cells in chronically HCV-infected patients. For these experiments, CD4$^+$CD25$^+$ Treg cells as well as CD4$^+$CD25$^-$ cells were purified from the PBMC of five chronically HCV-infected patients, five persons with resolved HCV infection, and five healthy blood donors. These cells were added back to the CD4-depleted PBMC prior

to peptide stimulation. Interestingly, as shown in Fig. 5A, the suppression of influenza virus-specific CD8$^+$ T-cell responses was similar to the suppression of HCV-specific CD8$^+$ T-cell responses. These results suggest that the Treg-mediated suppression of virus-specific CD8$^+$ T-cell responses is not limited to the persisting virus.

Next, we tested if the suppression of virus-specific CD8$^+$ T-cell responses is specific for chronic HCV infection. To address this issue we analyzed the Treg-mediated suppression of HCV- and influenza virus-specific CD8$^+$ T-cell responses in persons with resolved HCV infection (Table 1) and of influenza virus-specific CD8$^+$ T-cell responses in healthy blood donors. Interestingly, the Treg-mediated suppression of both HCV ($P = 0.018$) and influenza virus ($P = 0.028$)-specific CD8$^+$ T-cell proliferation was significantly less in individuals with resolved HCV infection. In addition, suppression of influenza virus-specific CD8$^+$ T-cell proliferation in healthy blood donors was comparable to that of persons with resolved HCV infection and less compared to chronically HCV-infected patients ($P = 0.018$) (Fig. 5A). These results suggest that chronic HCV infection leads to an expansion of Treg cells that suppress antiviral CD8$^+$ T-cell responses irrespective of their antigen specificity. In this regard, it is also important to note that higher numbers of CD4$^+$CD25$^+$ cells were detectable in chronically HCV-infected patients compared to persons with resolved HCV infection or healthy blood donors (Fig. 5B).

DISCUSSION

Impaired proliferative, cytokine and cytotoxic effector functions of HCV-specific CD8⁺ T cells are a hallmark of chronic HCV infection (14, 43, 47). The dysfunctions of HCV-specific CD8⁺ T cells probably contribute significantly to viral persistence. The mechanisms responsible for these dysfunctions of HCV-specific T cells in chronically infected patients are still not understood. Several explanations have been proposed, including HCV variants with altered epitope sequences, induction of anergy by high antigen levels, the lack of different helper functions, or the suppressive activity of Treg cells (33).

Our study shows at a single epitope level with well-described HLA-A2 epitopes that CD4⁺CD25⁺ T cells contribute to the dysfunction of virus-specific CD8⁺ T cells, such as proliferation and IFN-γ production, during chronic HCV infection. Indeed, by investigating the Treg-mediated suppression of HCV-specific CD8⁺ T cells on a single-cell basis with CFSE staining, we could show that proliferation is inhibited in a dose-dependent manner. In addition, we showed that Treg-mediated inhibition of HCV-specific CD8⁺ T-cell proliferation requires direct cell-cell contact and is independent of IL-10 and TGF-β.

The biological role of regulatory T cells in chronic HCV infection is further supported by our observation that regulatory T cells from patients recovered from HCV infection exhibited significantly less suppressive activity. These results support the hypothesis that chronic viral infection leads to the induction of suppressive Treg cells that inhibit antiviral immune responses (1, 16, 25, 36). The high frequency of CD4⁺CD25⁺ T cells observed in chronically HCV-infected patients further suggests an important role for regulatory T cells in HCV persistence (36) (Fig. 5B). Indeed, the generation of adaptive regulatory T cells may be a normal process that occurs to prevent immunopathological damage and thereby contributes to viral persistence. It is important to note that although CD25 is expressed on most regulatory T cells, it is not specific since it can also be expressed on activated CD4⁺ T cells. Recently, Foxp3 has been shown to be a good marker for CD4⁺CD25⁺ T cells in mice, however, its relevance in humans has not been clearly defined so far (10, 28).

Importantly, our results indicate that the Treg-mediated suppression in chronically HCV-infected patients is not restricted to HCV-specific CD8⁺ T cells but also involves other virus-specific CD8⁺ T cells. Indeed, we observed a similar suppression of HCV- and influenza virus-specific CD8⁺ T cells in chronically HCV-infected patients (Fig. 5A). In contrast, the suppression of influenza virus-specific CD8⁺ T-cell responses was significantly less in persons with resolved HCV infection and healthy blood donors. These results suggest that chronic HCV infection may lead to an unspecific activation and expansion of naturally occurring Treg cells or the induction of antigen-specific Treg cells (38, 39, 46) that regulate immune responses not only towards the ligands they were selected for but also towards other antigens in a bystander manner. This possibility is supported by results from in vitro models showing that Treg cells require activation via their T-cell receptor to become suppressive, but once activated, they can perform their suppressor function completely nonspecifically (15, 42). In addition, Suvas et al. (37) observed that acute herpes simplex virus infection resulted in enhanced Treg function, leading to the suppression of CD8⁺ T-cell responses to both viral and unrelated antigens and indicating an antigen-nonspecific bystander inhibition of CD8⁺ T cells.

It is important to note that the similar suppression of HCV- and influenza virus-specific CD8⁺ T cells observed in vitro may not necessarily reflect the situation in vivo. Indeed, it is well conceivable that Treg cells may be activated and perform their suppressive effects in vivo primarily in compartments with high antigen load, such as the virus-infected liver. This would ensure that the suppressive activity is limited to the biologically important compartment. Indeed, the accumulation of suppressive Treg cells at the site of disease has already been demonstrated in other models (3, 13, 35). Thus, the ratio of virus-specific CD8⁺ T cells versus CD4⁺CD25⁺ regulatory T cells that is required for a strong suppression (ratios 3:1 to 10:1, Fig. 3) in our study might indeed mirror the intrahepatic situation. In order to address this important question in chronic HCV infection, a further characterization of the function and phenotype of Treg cells in the liver of chronically HCV-infected patients is needed.

The data of the present paper suggest that the Treg-mediated suppression of HCV-specific CD8⁺ T-cell functions is enhanced in the chronic phase of infection. Indeed, by comparing the Treg-mediated suppression of HCV-specific CD8⁺ T cells in patients with chronic versus persons with resolved HCV infection, we observed a significantly lower suppression of HCV-specific CD8⁺ T-cell proliferation in persons with resolved HCV infection despite using the same number of CD4⁺CD25⁺ T cells. These results suggest that the different degrees of suppressive activity of Treg cells may depend on numerous yet unknown factors that are induced during chronic HCV infection. Thus, our findings suggest that the Treg-mediated impairment of HCV-specific CD8⁺ T-cell proliferation and IFN-γ secretion is specific for the chronic phase of infection.

These observations are in agreement with previous reports describing impaired effector functions of HCV-specific CD8⁺ T cells in chronically infected patients (14, 43, 47). It is important to note that dysfunctions can also be observed in acutely infected patients. However, most studies have suggested that the impaired CD8⁺ T-cell effector functions are only transient during acute resolving infection (23, 41, 44) because HCV-specific CD8⁺ T cells can progressively improve their function in patients with self-limited hepatitis C, while the CD8 function remains persistently depressed in subjects with a chronic evolution (44).

Our finding that depletion of CD4⁺ cells leads to an increase of virus-specific CD8⁺ T cells (Fig. 1) is somewhat paradoxical since it is widely assumed that CD4⁺ cells are required for the priming, induction, and maintenance of virus-specific CD8⁺ T-cell responses (18). Several groups have shown that peripheral and intrahepatic HCV-specific CD4⁺ T-cell responses are associated with viral clearance in acutely infected humans and chimpanzees (8, 11, 26, 40). In addition, a recent depletion study of CD4⁺ cells in chimpanzees has clearly demonstrated that CD4⁺ cells are required for effective antiviral CD8⁺ T-cell immunity since HCV persistence and immune evasion occurred in the absence of CD4⁺ T-cell help in two previously immune animals (12). While these studies support an important role for CD4⁺ T cells in the outcome of HCV infection,

they have been performed in acutely infected patients or chimpanzees.

By contrast, our study focused on chronic HCV infection where HCV-specific CD4$^+$ T-cell responses are usually weak, monospecific, and dysfunctional (43). Our results favor a model in which HCV-specific CD4$^+$ cells have two very different functions during the course of HCV infection. In the acute phase of infection, CD4$^+$ helper T cells contribute to the induction and maintenance of a functional CD8$^+$ T-cell response. In the chronic phase, however, Treg cells suppress virus-specific CD8$^+$ T-cell responses and thereby help the virus to persist. In future studies it will be important to determine at what time point during infection Treg cells become active in HCV-infected patients. It is intriguing to speculate, however, that the induction of Treg cells is an early event that may be responsible for the early decrease of virus-specific CD4$^+$ and CD8$^+$ T-cell responses observed in some acutely infected patients developing viral persistence (11, 22, 41).

The importance and biological relevance of our study are strengthened by the fact that other groups have also recently observed a suppression of virus-specific CD8$^+$ T-cell proliferation by regulatory T cells during persistent hepatitis C virus infection (5, 32a). Furthermore, Cabrera et al. detected a higher proportion of CD4$^+$CD25$^+$ T cells in chronic HCV infection and that these cells secrete IL-10 and TGF-β. Interestingly, in this study the Treg-mediated suppression of HCV-specific IFN-γ production by PBMCs could be recovered by the addition of anti-TGF-β (5). In contrast, Rushbrook et al. and we did not observe that the Treg-mediated inhibition of proliferation was abolished by the addition of anti-TGF-β suggesting that different effector functions of virus-specific CD8$^+$ T cells might be controlled by different mechanisms. Finally, Rushbrook et al. also observed that regulatory T cells did not only suppress HCV-specific, but also Epstein-Barr virus- and cytomegalovirus-specific CD8$^+$ T-cell responses in chronically HCV-infected patients also indicating that the Treg-mediated suppressive activity is not limited to virus-specific CD8$^+$ T cells targeting the persisting virus (32a).

In summary, our results suggest that the presence of immunosuppressive CD4$^+$ Treg cells accounts at least in part for impaired proliferation of virus-specific CD8$^+$ T cells during chronic HCV infection. The Treg-mediated suppression is dose dependent, requires direct cell-cell contact, is independent of IL-10 and TGF-β, and is not restricted to virus-specific CD8$^+$ T cells targeting the persisting virus but also involves other virus-specific CD8$^+$ T cells. These findings may have important implications for the development of new immunotherapeutic strategies against chronic HCV infection via stimulation of HCV-specific immunity through inhibition or depletion of Treg cells.

ACKNOWLEDGMENTS

REFERENCES

1. **Aandahl, E. M., J. Michaelsson, W. J. Moretto, F. M. Hecht, and D. F. Nixon.** 2004. Human CD4$^+$ CD25$^+$ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. J. Virol. **78:**2454–2459.
2. **Accapezzato, D., V. Francavilla, M. Paroli, M. Casciaro, L. V. Chircu, A. Cividini, S. Abrignani, M. U. Mondelli, and V. Barnaba.** 2004. Hepatic expansion of a virus-specific regulatory CD8$^+$ T-cell population in chronic hepatitis C virus infection. J. Clin. Investig. **113:**963–972.
3. **Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks.** 2002. CD4$^+$CD25$^+$ regulatory T cells control Leishmania major persistence and immunity. Nature **420:**502–507.
4. **Bluestone, J. A., and A. K. Abbas.** 2003. Natural versus adaptive regulatory T cells. Nat. Rev. Immunol. **3:**253–257.
5. **Cabrera, R., Z. Tu, Y. Xu, R. J. Firpi, H. R. Rosen, C. Liu, and D. R. Nelson.** 2004. An immunomodulatory role for CD4$^+$CD25$^+$ regulatory T lymphocytes in hepatitis C virus infection. Hepatology **40:**1062–1071.
6. **Camara, N. O., F. Sebille, and R. I. Lechler.** 2003. Human CD4$^+$CD25$^+$ regulatory cells have marked and sustained effects on CD8$^+$ T-cell activation. Eur. J. Immunol. **33:**3473–3483.
7. **Chang, K. M., R. Thimme, J. J. Melpolder, D. Oldach, J. Pemberton, J. Moorhead-Loudis, J. G. McHutchison, H. J. Alter, and F. V. Chisari.** 2001. Differential CD4 and CD8 T-cell responsiveness in hepatitis C virus infection. Hepatology **33:**267–276.
8. **Diepolder, H. M., R. Zachoval, R. M. Hoffmann, E. A. Wierenga, T. Santantonio, M. C. Jung, D. Eichenlaub, and G. R. Pape.** 1995. Possible mechanism involving T-lymphocyte response to nonstructural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet **346:**1006–1007.
9. **Dittmer, U., H. He, R. J. Messer, S. Schimmer, A. R. Olbrich, C. Ohlen, P. D. Greenberg, I. M. Stromnes, M. Iwashiro, S. Sakaguchi, L. H. Evans, K. E. Peterson, G. Yang, and K. J. Hasenkrug.** 2004. Functional impairment of CD8$^+$ T cells by regulatory T cells during persistent retroviral infection. Immunity **20:**293–303.
10. **Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky.** 2003. Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. Nat. Immunol. **4:**330–336.
11. **Gerlach, J. T., H. M. Diepolder, M. C. Jung, N. H. Gruener, W. W. Schraut, R. Zachoval, R. Hoffmann, C. A. Schirren, T. Santantonio, and G. R. Pape.** 1999. Recurrence of hepatitis C virus after loss of virus-specific CD4$^+$ T- cell response in acute hepatitis C. Gastroenterology **117:**933–941.
12. **Grakoui, A., N. H. Shoukry, D. J. Woollard, J. H. Han, H. L. Hanson, J. Ghrayeb, K. K. Murthy, C. M. Rice, and C. M. Walker.** 2003. HCV persistence and immune evasion in the absence of memory T-cell help. Science **302:**659–662.
13. **Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo.** 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature **389:**737–742.
14. **Gruener, N. H., F. Lechner, M. C. Jung, H. Diepolder, T. Gerlach, G. Lauer, B. Walker, J. Sullivan, R. Phillips, G. R. Pape, and P. Klenerman.** 2001. Sustained dysfunction of antiviral CD8$^+$ T lymphocytes after infection with hepatitis C virus. J. Virol. **75:**5550–5558.
15. **Homann, D., A. Holz, A. Bot, B. Coon, T. Wolfe, J. Petersen, T. P. Dyrberg, M. J. Grusby, and M. G. von Herrath.** 1999. Autoreactive CD4+ T cells protect from autoimmune diabetes via bystander suppression using the IL-4/Stat6 pathway. Immunity **11:**463–472.
16. **Iwashiro, M., R. J. Messer, K. E. Peterson, I. M. Stromnes, T. Sugie, and K. J. Hasenkrug.** 2001. Immunosuppression by CD4+ regulatory T cells induced by chronic retroviral infection. Proc. Natl. Acad. Sci. USA **98:** 9226–9230.
17. **Jonuleit, H., E. Schmitt, H. Kakirman, M. Stassen, J. Knop, and A. H. Enk.** 2002. Infectious tolerance: human CD25$^+$ regulatory T cells convey suppressor activity to conventional CD4$^+$ T helper cells. J. Exp. Med. **196:**255–260.
18. **Kalams, S. A., and B. D. Walker.** 1998. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. J. Exp. Med. **188:** 2199–2204.
19. **Kinter, A. L., M. Hennessey, A. Bell, S. Kern, Y. Lin, M. Daucher, M. Planta, M. McGlaughlin, R. Jackson, S. F. Ziegler, and A. S. Fauci.** 2004. CD25$^+$CD4$^+$ regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4$^+$ and CD8$^+$ HIV-specific T-cell immune responses in vitro and are associated with favorable clinical markers of disease status. J. Exp. Med. **200:**331–343.
20. **Koziel, M. J., D. Dudley, J. T. Wong, J. Dienstag, M. Houghton, R. Ralston, and B. D. Walker.** 1992. Intrahepatic cytotoxic T lymphocytes specific for hepatitis C virus in persons with chronic hepatitis. J. Immunol. **149:**3339–3344.
21. **Lauer, G. M., and B. D. Walker.** 2001. Hepatitis C virus infection. N. Engl. J. Med. **345:**41–52.
22. **Lechner, F., N. H. Gruener, S. Urbani, J. Uggeri, T. Santantonio, A. R. Kammer, A. Cerny, R. Phillips, C. Ferrari, G. R. Pape, and P. Klenerman.** 2000. CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. Eur. J. Immunol. **30:**2479–2487.
23. **Lechner, F., D. K. Wong, P. R. Dunbar, R. Chapman, R. T. Chung, P. Dohrenwend, G. Robbins, R. Phillips, P. Klenerman, and B. D. Walker.** 2000. Analysis of successful immune responses in persons infected with hepatitis C virus. J. Exp. Med. **191:**1499–1512.

24. **Lyons, A. B.** 2000. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. J. Immunol. Methods **243:** 147–154.

25. **MacDonald, A. J., M. Duffy, M. T. Brady, S. McKiernan, W. Hall, J. Hegarty, M. Curry, and K. H. Mills.** 2002. CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons. J Infect. Dis. **185:**720–727.

26. **Missale, G., R. Bertoni, V. Lamonaca, A. Valli, M. Massari, C. Mori, M. G. Rumi, M. Houghton, F. Fiaccadori, and C. Ferrari.** 1996. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. J. Clin. Investig. **98:** 706–714.

27. **Mittrucker, H. W., and S. H. Kaufmann.** 2004. Mini-review: regulatory T cells and infection: suppression revisited. Eur. J. Immunol. **34:**306–312.

28. **Morgan, M. E., J. H. van Bilsen, A. M. Bakker, B. Heemskerk, M. W. Schilham, F. C. Hartgers, B. G. Elferink, L. van der Zanden, R. R. de Vries, T. W. Huizinga, T. H. Ottenhoff, and R. E. Toes.** 2005. Expression of FOXP3 mRNA is not confined to CD4$^+$CD25$^+$ T regulatory cells in humans. Hum. Immunol. **66:**13–20.

29. **Ng, W. F., P. J. Duggan, F. Ponchel, G. Matarese, G. Lombardi, A. D. Edwards, J. D. Isaacs, and R. I. Lechler.** 2001. Human CD4$^+$CD25$^+$ cells: a naturally occurring population of regulatory T cells. Blood **98:**2736–2744.

30. **O'Garra, A., and P. Vieira.** 2004. Regulatory T cells and mechanisms of immune system control. Nat. Med. **10:**801–805.

31. **Piccirillo, C. A., and E. M. Shevach.** 2001. Cutting edge: control of CD8+ T-cell activation by CD4+CD25+ immunoregulatory cells. J. Immunol. **167:** 1137–1140.

32. **Rehermann, B., K. M. Chang, J. G. McHutchison, R. Kokka, M. Houghton, and F. V. Chisari.** 1996. Quantitative analysis of the peripheral blood cytotoxic T lymphocyte response in patients with chronic hepatitis C virus infection. J. Clin. Investig. **98:**1432–1440.

32a. **Rushbrook, S. M., S. M. Ward, E. Unitt, S. L. Vowler, M. Lucas, P. Klenerman, and G. J. M. Alexander.** 2005. Regulatory T cells suppress in vitro proliferation of virus-specific CD8$^+$ T cells during persistent hepatitis C virus infection. J. Virol. **79:**7852–7859.

33. **Sakaguchi, S.** 2000. Regulatory T cells: key controllers of immunologic self-tolerance. Cell **101:**455–458.

34. **Shevach, E. M.** 2002. CD4+ CD25+ suppressor T cells: more questions than answers. Nat. Rev. Immunol. **2:**389–400.

35. **Steinbrink, K., E. Graulich, S. Kubsch, J. Knop, and A. H. Enk.** 2002. CD4$^+$ and CD8$^+$ anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. Blood **99:**2468–2476.

36. **Sugimoto, K., F. Ikeda, J. Stadanlick, F. A. Nunes, H. J. Alter, and K. M. Chang.** 2003. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. Hepatology **38:** 1437–1448.

37. **Suvas, S., U. Kumaraguru, C. D. Pack, S. Lee, and B. T. Rouse.** 2003. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T-cell responses. J. Exp. Med. **198:**889–901.

38. **Taams, L. S., M. Vukmanovic-Stejic, J. Smith, P. J. Dunne, J. M. Fletcher, F. J. Plunkett, S. B. Ebeling, G. Lombardi, M. H. Rustin, J. W. Bijlsma, F. P. Lafeber, M. Salmon, and A. N. Akbar.** 2002. Antigen-specific T-cell suppression by human CD4+CD25+ regulatory T cells. Eur. J. Immunol. **32:**1621–1630.

39. **Tanchot, C., F. Vasseur, C. Pontoux, C. Garcia, and A. Sarukhan.** 2004. Immune regulation by self-reactive T cells is antigen specific. J. Immunol. **172:**4285–4291.

40. **Thimme, R., J. Bukh, H. C. Spangenberg, S. Wieland, J. Pemberton, C. Steiger, S. Govindarajan, R. H. Purcell, and F. V. Chisari.** 2002. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. Proc. Natl. Acad. Sci. USA **99:**15661–15668.

41. **Thimme, R., D. Oldach, K. M. Chang, C. Steiger, S. C. Ray, and F. V. Chisari.** 2001. Determinants of viral clearance and persistence during acute hepatitis C virus infection. J. Exp. Med. **194:**1395–1406.

42. **Thornton, A. M., and E. M. Shevach.** 2000. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. J. Immunol. **164:**183–190.

43. **Ulsenheimer, A., J. T. Gerlach, N. H. Gruener, M. C. Jung, C. A. Schirren, W. Schraut, R. Zachoval, G. R. Pape, and H. M. Diepolder.** 2003. Detection of functionally altered hepatitis C virus-specific CD4 T cells in acute and chronic hepatitis C. Hepatology **37:**1189–1198.

44. **Urbani, S., C. Boni, G. Missale, G. Elia, C. Cavallo, M. Massari, G. Raimondo, and C. Ferrari.** 2002. Virus-specific CD8$^+$ lymphocytes share the same effector-memory phenotype but exhibit functional differences in acute hepatitis B and C. J. Virol. **76:**12423–12434.

45. **Vigouroux, S., E. Yvon, E. Biagi, and M. K. Brenner.** 2004. Antigen-induced regulatory T cells. Blood **16:**16.

46. **Wang, H. Y., D. A. Lee, G. Peng, Z. Guo, Y. Li, Y. Kiniwa, E. M. Shevach, and R. F. Wang.** 2004. Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy. Immunity **20:**107–118.

47. **Wedemeyer, H., X. S. He, M. Nascimbeni, A. R. Davis, H. B. Greenberg, J. H. Hoofnagle, T. J. Liang, H. Alter, and B. Rehermann.** 2002. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. J. Immunol. **169:**3447–3458.